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Identification of Biomarkers Associated with the Healing of Chronic Wounds

PRINCIPAL INVESTIGATOR:
Laura E. Edsberg, Ph.D.

CONTRACTING ORGANIZATION:
Daemen College
Amherst, NY 14226

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Laura E. Edsberg, Ph.D.

Email: ledsberg@daemen.edu

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14. ABSTRACT

It is the objective of this study to identify the biomarkers associated with the earliest stages of healing in chronic wounds and the biochemical differences in the burn fluid of burns with hypertrophic scarring and those without. The findings of this study are intended to facilitate the development a diagnostic tools, which could be used to evaluate the healing process. Samples have been collected from interior and peripheral wound sites over 6 weeks from 50 subjects and analyzed using 2D Page, iTRAQ$_{TM}$, and antibody arrays. Different proteins have been identified in healed versus chronic wounds and in different locations within the wounds. The correlation of wound biochemistry, clinical appearance, and clinical outcome is critical to the understanding of pressure ulcer healing. These findings will aid in the development of criteria for evaluating the healing process and response to treatment

15. SUBJECT TERMS
Chronic wound, burn wounds, healing, hypertrophic scarring 2D Page, iTRAQ$_{TM}$, antibody arrays

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INTRODUCTION

The fact that there are differences in chronic versus normal healing wounds is well documented. What is unknown at this time are the specific biomarkers associated with healing wounds, the role each of these biomarkers play in wound healing, and the biomarkers that can serve as the earliest predictors of healing. It is our hypothesis that specific cytokines, proteases, and growth factors serve as the earliest indicators of healing in chronic wounds. The initial objective of this study was to identify the biomarkers associated with the earliest stages of healing in chronic wounds. Hypertrophic scarring is a common complication associated with healed deep burns. It is our hypothesis that specific quantifiable biochemical differences in the sera and burn fluid exist between burn patients that develop hypertrophic scarring and those that do not. The objectives of this continuation were to evaluate the biochemical profiles of healing burns and compare those with hypertrophic scarring with those without. The findings of this study are intended to facilitate the development a diagnostic tool, which would assist in the evaluation of the healing process in chronic and burn wounds.

BODY

Statement of Work

Technical Objective 1: To identify the biochemical changes that occur as a chronic wound begins the healing process.

a. Analyze fluid samples to determine proteins present
b. Identify differences between subjects and subject time points
c. Confirm protein identities

Technical Objective 2: To assess the rate of healing of the wounds analyzed.

a. Measure wound
b. Calculate trajectories of healing for wounds over time

Technical Objective 3: To evaluate the location of the biomarkers assessed.

a. Compare proteins found in different locations using protein analysis

Technical Objective 4: To identify the earliest changing biomarkers occurring in wounds which progressed toward healing.

a. Correlate the changes in wound chemistry with the rate of healing

b. Analyze the earliest biochemical changes present

Technical Objectives 1,2,3, & 4:

As of June 14, 2008 enrollment of the study was closed. 121 subjects were screened and 50 were enrolled.

Technical Objective 1:

The analysis of wound fluid samples to determine the proteins present is completed. The methodology developed to screen the samples for potential targets and analyze and quantify the proteins of interest consists of antibody arrays and multiplexed isobaric tagging technology (iTRAQ™). iTRAQ identifies the most abundant proteins present and the least abundant include most cytokines, which is the majority of what has been previously reported in the literature regarding the biochemistry of pressure ulcers and other types of wounds. As a result, the inclusion of antibody arrays allows samples to be analyzed for the less abundant and smaller proteins.

Using two-dimensional polyacrlyamide gel electrophoresis (2D-gel) it was found that healed wounds showed an increased number of spots coincident with wound closure, while unhealed wounds showed no temporal trend (Wyffels JT, Fries KM, Randall J, Ha D, Lodwig C, Brogan M, Shero M, Edsberg LE. Analysis of chronic pressure ulcer wound fluid using two-dimensional electrophoresis. International Wound Journal 2010;7:236-248.).Ultimately 2D-gel proved to be a less than ideal method to search for biomarkers in chronic wound fluid. Though this 2D proteomic approach has proven successful for biomarker discovery in other systems, because of the high numbers of spots present in gels, as well as the complexity of spots, high cost of protein identification from gel plugs and sample consumption, further biomarker identification via 2D technology was abandoned in favor other techniques including iTRAQ and label-based microarrays that simultaneously measure a panel of proteins for a more complete assessment of the proteome.

282 proteins were identified in wound samples using iTRAQ. The relative change in protein amount as compared to other samples/days has been analyzed for the samples. The iTRAQ data were analyzed using Ingenuity Pathway Analysis Software (IPA), a software package that allows analysis, modeling, and literature searches for similar proteins. The data was also analyzed utilizing bioinformatics by The Wistar Institute Philadelphia, PA.

Technical Objective 2:

To assess the rate of healing, the wounds were photographed and their area calculated at each time point. As previously reported, all wounds have been separated by clinical outcome into healed, healing, and chronic categories based on area measurements over the 42 days. Wounds that had a 81-100% decrease in area are categorized as healed, wounds with a 40-80% decrease in size are healing, and wounds with a less than 39% decrease in size or an increase in size were labeled chronic. As previously reported, wound area versus the time point has been graphed as wound trajectories. The manuscript based on the models tested for wound measurement and clinical outcome prediction is in preparation and will be submitted by September 2010. Tissue type calculations based on the method previously reported were completed and final correlation with clinical outcome and tissue type has been evaluated. No correlation between tissue type present and wound outcome was found. The presence of granulation tissue is a positive clinical sign in the clinical assessment of a chronic wound bed, but the presence of granulation tissue did not correlate with healing in chronic wounds (Manuscript submitted to Advances in Skin and Wound care August 2010). The tissue type and clinical outcome correlation was examined as part of the search for potential biomarkers of healing. In previous reports the correlation of biochemical changes to changes in tissue type were noted. While these changes may occur, their lack of correlation with outcome makes these biochemical changes a poor choice for potential biomarkers.

Technical Objective 3:

To evaluate the location of the biomarkers assessed, samples were collected from both peripheral and interior locations on each wound at each time point. Antibody array data and

iTRAQ data have shown differences in the molecules present and the location within the wound. Molecules associated with cell death were not found in internal sites of healed wounds, but were present in some peripheral locations. Large numbers of molecules associated with cell death present in both internal and peripheral samples from chronic wounds. The differences in proteins present in peripheral and internal sites of both healed and chronic wounds was also confirmed by significant differences in spot distribution in internal versus peripheral locations within wounds.

Technical Objective 4:

Custom arrays were developed based on the 2D and iTRAQ findings and the final set of custom antibody arrays included calreticulin, ENO1, Gelsolin, Progranulin, sRAGE. S100A12/ENRAGE, S100A6, S100A7, S100A8, and S100A9 as targets.

The bioinformatics analysis of the iTRAQ and microarray data is as follows and in Appendix A:

To compare protein levels of interior and periphery samples, the interior and periphery samples from the same wound that measured at the identical day were paired, and their differences in protein levels were considered as new observations. Both Wilcoxon test and t-test were performed and compared. In this analysis, Wilcoxon test is more appropriate than t-test due to the following reasons:

(a) there is no evidence to assume normal populations;

(b) the sample distribution does not look a normal distribution;

(c) the sample size is not large enough.

Hence we only use the results of Wilcoxon test to find proteins whose level were significantly different (at the significance level $\alpha = 0.1$) between interior and periphery samples.

To compare healed and chronic samples, we first did not consider any time information and treat all data points as independent samples. We performed 2 sample t-test and Mann-Whitney test. T-test is the most common method and the most powerful test when certain conditions are satisfied. However, in this case, Mann-Whitney test provided better results because the sample size is relatively small and the distributions may not be normal distributions.

Next, we compared the trend of protein level between healed and chronic samples during time evolution by building a linear mixed model for each protein. The exact mathematical model is set as

$$P_{ijt} = \mu + \alpha_i + \beta t + \gamma_i t + e_{ijt},$$

where P_{ijt} stands for the protein level of the i[th] group (i=1 for healed sample and i=2 for chronic sample) and the j[th] subject at time t. μ is the protein level at time t=0, and β explains the increment/decrement of the protein level when time changes 1 unit (1 day in this data) in the healed sample. Both α_i and γ_i explain the difference in protein levels of healed/chronic samples. For identifiability of the model, we set $\alpha_1 = \gamma_1 = 0$. The last term e_{ijt} stands for the error term, which considers the natural variation of the measurement. This model can be re-written as

$$P_{ijt} = \begin{cases} (\mu + 0) + (\beta + 0)t + e_{1jt}, & for\ healed\ sample\ (i = 1) \\ (\mu + \alpha_2) + (\beta + \gamma_2)t + e_{2jt}, & for\ chronic\ sample\ (i = 2). \end{cases}$$

In this model, we assume that the effect of time on protein level is linear, and the intercept and slope of time effect are different by group. In addition, this model reflects the dependency of observations from the same subject.

One thing to be notice here is how we re-define "time" in this analysis. We set the last observation day as time = 0, and compute the relative time for the other observation days. The reasons are as follows:

(a) Day 0 in the original data set means the beginning of the observation, not the time when the wound occurs, thus day0 in different wounds are actually at different stages;

(b) In healed sample, the last day of the observation means the day when the wound is completely healed;

(c) In chronic sample, we do not have the beginning and the end stages of the wound, so the transformation of the time does not change anything.

Bioinformatics Results

iTRAQ Data Analysis

To compare protein level between interior samples vs. periphery samples, only those interior and periphery samples from the same wound that measured at the identical day in one iTRAQ experiments were considered. Four chronic wounds on seven days were considered in this analysis (BM015 on day42, BM017 on day35, BM026 on day14, BM029 on day2, day10, day14, and day21). We have identified ten proteins that four of them consistently have lower protein level in periphery samples than interior samples and the rest have higher protein level in periphery samples than interior samples (Table1).

Table1. ITRAQ Ratio between periphery and interior samples (P value are in the parenthesis)

Protein	BM029-	BM029-	BM029-	BM026-	BM029-	BM017-	BM015-

	Day2	Day10	Day14	Day14	Day21	Day35	Day42
PKM2	0.27(0.10)	0.6(0.13)	0.16(0.01)	0.26(0.25)	0.19(0.00)	0.79(0.00)	0.67(0.39)
PFN1	0.45(0.15)	0.79(0.18)	0.72(0.99)	0.11(0.80)	2.5(0.13)	0.65(0.00)	0.07(0.00)
IGLC1	0.49(0.12)	0.60(0.82)	0.86(0.74)	0.05(0.05)	0.48(0.20)	0.86(0.00)	1.21(0.44)
IGHG1	0.18(0.02)	0.23(0.45)	1.10(0.84)	0.69(0.70)	0.67(0.24)	0.95(0.02)	1.10(0.98)
KRT6A	18.54(0.00)	14.32(0.02)	4.97(0.12)	NA	5.86(0.02)	2.29(0.00)	NA
KRT14	19.59(0.00)	8.95(0.18)	7.87(0.21)	NA	9.29(0.22)	3.54(0.00)	2.99(0.22)
S100A7	14.86(0.11)	99.08(0.03)	17.54(0.12)	NA	29.65(0.03)	4.79(0.00)	46.99(0.04)
SERPINA1	21.88(0.01)	4.06(0.04)	3.28(0.00)	0.82(0.76)	0.98(0.87)	1.28(0.00)	1.13(0.71)
HBA1	1.74(0.74)	4.70(0.00)	5.01(0.20)	8.17(0.06)	3.87(0.39)	1.01(0.57)	3.47(0.00)
HBB	1.47(0.84)	4.06(0.00)	6.85(0.07)	0.28(0.83)	3.63(0.22)	1.02(0.75)	6.61(0.00)

To identify the trend of protein level during healing process on healed wounds, we considered the iTRAQ ratios of adjacent time points and compared among healed wounds. However, no consistent trend could be identified in both interior and periphery samples.

Protein microarray analysis

To compare protein level between interior samples and periphery samples, only chronic wounds were considered due to the lack of periphery samples in healed wounds. To obtain the list of proteins shown in Table 2 below, we used only the result of Wilcoxon test because of the reasons described in Method section. In this analysis, seven proteins have lower protein level in periphery samples than interior samples, and three proteins have higher protein level in periphery samples than interior samples including S100A7, which has also been identified in iTRAQ data.

Table2. Median difference between periphery and interior samples

Protein	Median (P-I)	P Value	Sample Size
G-CSF	-	0.02	14
ICAM-1	-	0.07	14
IL-6	-	0.08	14
MIP-1b	-	0.06	14
MMP-2	-	0.02	14
MMP-3	-	0.09	14
TIMP-2	-	0.08	14
sRAGE	+	0.09	16
S100A6	+	0.04	16
S100A7	+	0.03	16

If we treat each observation as an independent sample and do not consider any time information, the protein levels of healed and chronic samples can be compared using 2 sample t-test or Mann-Whitney test. There are 18 healed interior samples and 21 chronic interior samples in both HI3 array and MMP array; and 21 healed interior samples and 23 chronic interior samples in customer array. We displayed only the results of Mann-Whitney tests (at the significance level $\alpha = 0.1$) (Table3) because t-test and Mann-Whitney test gave quite similar results. Along with p-values, we used the term “direction” to interpret that the protein level in the healed samples tends to be lower or higher than those in the chronic samples. For

periphery samples, the number of healed samples was too small to identify a protein that is significantly different between healed and chronic wounds.

Table3. Comparison of the healed vs. chronic wounds

Protein	Array	Direction	Pvalue
Eotaxin -2	HI3	H > C	0.00
ICAM-1	HI3	H > C	0.03
IL-16	HI3	H > C	0.00
MIP-1d	HI3	H > C	0.04
GM-CSF	HI3	H < C	0.06
I-309	HI3	H < C	0.03
IFNg	HI3	H < C	0.01
IL-1a	HI3	H < C	0.00
IL-1b	HI3	H < C	0.00
IL-8	HI3	H < C	0.00
IL-11	HI3	H < C	0.06
IL-12p40	HI3	H < C	0.02
IL-15	HI3	H < C	0.00
TIMP-1	HI3	H < C	0.02
TIMP-2	HI3	H < C	0.00
TNF RI	HI3	H < C	0.00
TNF RII	HI3	H < C	0.00
MMP-3	MMP	H > C	0.01
MMP-10	MMP	H > C	0.00
MMP-13	MMP	H > C	0.00
TIMP-1	MMP	H < C	0.03
TIMP-2	MMP	H < C	0.08

The independent assumption of the observation may not be realistic because protein levels were measured at several different time points from the same subject (the same wound), which results in some dependence structure. Therefore we also tried to reflect the dependency coming from repeated measurements using a mixed effect model. The result is not shown here because the dependent model identified much less significant proteins, which were also identified by Mann-Whitney test.

To compare the trend of protein level between healed and chronic samples during time evolution, a linear mixed model for each protein was built. Among 58 proteins (10 from Custom,

9 from MMP and 39 from HI3 arrays), only one protein gave significant parameter estimates of β, γ_i (Table4 and Fig1).

Table4. Comparison of the trend in healed vs. chronic wounds

Protein	μ		α_i		β		γ_i	
	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value
MIG	3540.28	.0001	-3321.23	.0457	97.49	.0011	-101.94	.0096

This result can be interpreted as MIG level increased 97.49 pg/mg per day in healed wounds, and decreased 4.45 (= 97.49-101.94) pg/mg per day in chronic wounds, which shows the significant difference time (linear) trend.

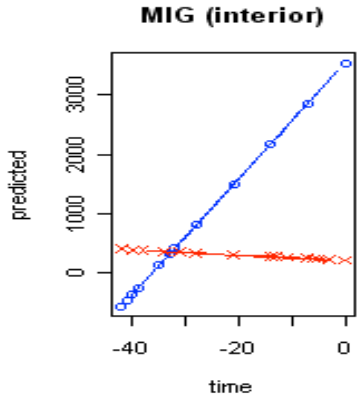


Figure1. Predicted protein levels in healed and chronic wounds by linear mix model. The y-axis is the predicted protein concentration (pg/mg) and the x-axis is the measured day. Blue line represents healed wounds and red line represents chronic wounds.

We have also tried other complicated models, such as quadratic models. However, none of those models were generated with successful result. Possibly it is due to the high individual variation of protein levels, compared to the small number of observations and the small between-group-variation. More results, such as proteins showing similar trends in both groups, can be found in Appendix A.

The current research is novel with respect to current published research in the field. There are no published studies characterizing of real-time surface biochemistry of pressure ulcers and no reported use of iTRAQ to analyze the proteome of pressure ulcer wound samples has been identified. Differential protein expression between healing and non-healing pressure ulcers has identified proteins, which may serve as indicators of wound healing. It is anticipated that some of the proteins identified will be significant with regard to our understanding of the healing of chronic wounds, as well as serving as potential biomarkers of healing. These biomarkers will serve as the basis of the development of an assay to predict wound outcome and may be the basis for future therapeutics developed to treat chronic wounds.

KEY RESEARCH ACCOMPLISHMENTS

- Developed methodology to map protein profiles of chronic wounds over time
- Identified proteins differences relative to wound location
- Utilized IPA for analysis of iTRAQ data
- Identified pathways correlated with proteins found in wounds in each category
- Identified proteins of interest for custom arrays
- Identified potential biomarkers of healing or lack of healing in chronic wounds

REPORTABLE OUTCOMES

- "Integrated Proteomic Analysis and siRNA Therapy for Treatment of Heterotropic Ossification." a new project based on the methodology developed with the current award, has been funded by the Department of Defense, U.S. Army Medical Research & Materiel Command (USAMRMC), Congressionally Directed Medical Research Programs. Idea Development Award. This project is a new collaboration with investigators from Rutgers University and the U.S. Army Institute of Surgical Research. This project was initiated after meeting at the June 23, 2009 Blood and Blood Safety PLR meeting.
- Herr M, Fries KM, Upton GL, Edsberg LE. Potential biomarkers of temporomandibular joint disorders. In Press, Journal of Oral & Maxillofacial Surgery. Research utilized methodology developed in current award.
- Wyffels JT, Fries KM, Randall J, Ha D, Lodwig C, Brogan M, Shero M, Edsberg LE. Analysis of chronic pressure ulcer wound fluid using two-dimensional electrophoresis. International Wound Journal 2010;7:236-248.
- "Proteins and Pressure Ulcer Outcomes", Chronic Wounds – Mechanisms and Diagnostics, 2nd Meeting of the Australian Wound and Tissue Repair Society, Perth, Western Australia, March 2010.
- "Translating Pressure Ulcer Research into Clinical Practice", Plenary Session – Research Translation, Journey into New Frontiers, Australian Wound Management Association, Perth, Western Australia, March 2010.
- Edsberg LE. "Proteomic Approaches for Studying the Phases of Wound Healing", Invited Chapter, Bioengineering Research of Chronic Wounds. Studies in

Mechanobiology, Tissue Engineering and Biomaterials Series. Gefen A (ed.), Springer-Verlag Berlin Heidelberg, 2009:343-362**.**

- "Role of Bioelectrical and Biochemical Fields in Chronic Non-Healing Wounds of People with Spinal Cord Injury", a new project based on the methodology developed with the current award, has been funded by the Ontario Neurotrauma Foundation for $211,154 for the period of 1/2008- 1/2010. This project will allow us to compare the biochemical profiles of pressure ulcers in people with and without spinal cord injuries. Additionally, biochemical changes after treatment with electrical stimulation will be analyzed. Enrollment is ongoing at this time.

- Abstract "Wound Surface Biochemistry of Healing and Non-Healing Pressure Ulcers", Edsberg LE, Fries KM, Brogan MS, Wyffels JT, poster presentation at the World Union of Wound Healing Societies, Third Congress, June 2008

- Invited presentation, "Potential Biomarkers of Healing and Non-Healing Pressure Ulcers", Edsberg LE, Plenary Session, 11th Annual European Pressure Ulcer Advisory Panel Meeting, Bruges, Belgium, September 2008.

- "Predicting Success in Wound Healing", Keynote Address, Edsberg LE, Care-Science and Practice, Tissue Viability Society Annual Conference, Llandudno, Wales, April 2009.

- Edsberg LE, Wyfells J. Correlation Between Protein Profiles and Tissue Types for Healing and Non-Healing Pressure Ulcers. European Wound Management Association, EWMA, Helsinki, Finland, May 2009.

CONCLUSION

The development of a methodology to identify the proteins present in chronic and healed wounds over time has been a major component in completion of the project. The utilization of iTRAQ$_{TM}$, antibody arrays, and bioinformatics to analyze the proteome allows a more complete analysis of the healing process over wounds over the course of time.

No studies have been identified using 2-D Page, iTRAQ™, and antibody arrays to characterize the environment of healed, healing, and non-healing pressure ulcers. The addition of the tissue type data further elucidates the biochemical profile of wounds. The correlation of wound biochemistry, clinical appearance, and clinical outcome is critical to understanding of pressure ulcer healing. These findings will aid in the development of criteria for evaluating the healing process and response to treatment. Ultimately, this work may serve as a basis for

profiling other types of wounds and for the development of therapies to treat wounds, which over time will decrease the suffering and deaths, as well as costs due to chronic wounds of all types.

STATEMENT OF WORK

Continuation

Burn Fluid and Patient Sera Biochemical Analysis as an Indicator of Aberrant Wound Repair and Hypertrophic Scarring

Phase I:

Technical Objective 1: Characterize the protein biochemistry of burn wounds.

a. Analyze wound fluid samples to determine proteins present
b. Identify trends present in burns as healing occurs

Technical Objective 2: Characterize the protein biochemistry in the sera of subjects with burn wounds.

a. Analyze sera to determine the proteins present
b. Identify trends present in subjects with burns during healing

Technical Objective 3: Assess the presence of hypertrophic scarring.

a. Burn Scar Index (Vancouver Scar Scale) parameters of scar will be assessed
b. Identify subjects with hypertrophic scarring burn wounds

Technical Objective 4: Correlate the differences between the sera and burn fluid samples during healing and identify biochemical differences between hypertrophic scarring and non-hypertrophic scarring subjects.

a. Correlate the trends in wound and sera biochemistry during healing
b. Correlate clinical outcome with biochemistry
c. Identify the differences present in sera and wound exudates in samples from subjects with hypertrophic scarring

Phase II:

Technical Objective 1: Develop a porcine model for burn wounds (second degree - superficial and deep).

a. Develop methods to reproducibly induce cutaneous thermal injuries in porcine tissue model.

b. Collect wound fluid from thermally injured swine for proteins of clinical interest, based upon those identified in Phase I of this project.

Technical Objective 2: Characterize the protein biochemistry of porcine wound fluids.

a. Analyze burn wound fluid by both ELISA and PIXIES.

b. Compare results from PIXIES with those from ELISA.

Technical Objective 3: Evaluate and validate porcine data with those obtained from Phase I studies.

a. Compare wound fluid biochemistry from thermally injured swine to that from normally-healing human wound fluid from Phase I of the study.

Phase I

Technical Objectives 1, 2, & 3:

The identification of a site to recruit and enroll subjects with burns has proven difficult. The Erie County Medical Center Burn Wound Unit has agreed to collaborate and the initial human subjects paperwork is being completed. The center's close proximity to the researchers is promising and collaborative research is part of the unit's mission.

Phase II

Technical Objective 1:

The domestic pig makes a suitable animal model because of the morphologic and functional similarities with human skin. One male 4 week old (at arrival) Yorkshire Cross pig was utilized for the study to date. After quarantine and a period of acclimation to human touch and interaction, the animal was 6 weeks old at the start of the experiment. Food and water were freely available but the animal was fasted overnight before any procedures. All procedures were done in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and regulations.

The animal was sedated and intubated endotracheally. The paravertebral area hair was shaved. Standardized partial-thickness burns were created by a heated 2cm x 2cm aluminum block wiped free of water just prior to application to prevent steam burn creation. Application pressure was through gravity alone and time was for 20 seconds for one set of burns, and 40 seconds for the second set. Each set of burns consisted of an untreated control, a burn covered with Silvadene cream, a burn covered with plain 0.5% HEMA hydrogel, and a burn covered with a KGF-doped HEMA 0.5% hydrogel.

Immediately following creation of the burns, the area was photographed using a digital camera, and re-photographed after all the dressings had been applied.

Phase II

Technical Objective 2:

Sensing of oxygen content of wounds using xerogel sensors was done by first taking a blank reading under low lighting conditions, then holding the chip containing the sensor in contact with the wound for 30 seconds under low light conditions. The fluorescence signal was then determined using a specially modified microscope.

The entire area surrounding each wound was painted with tissue adhesive, and all treated wounds were covered with a dressing. Pain medication and post-operative antibiotics were administered as indicated in the animal protocol.

Dressings were changed on the anesthetized animal on days 3 and 6. Prior to removal of the occlusive dressings, 20 µl of sterile PBS was injected under the dressing, and recovered along with any wound fluid that had accumulated. The fluid was frozen in liquid nitrogen for subsequent analysis for specific growth factors/cytokines. Spent hydrogels were removed and frozen at - 20°C for subsequent chemical analysis. The wounds were sensed for oxygen, followed by photography and re-dressed with the appropriate treatments and re-bandaged. Freshly sterilized/KGF-doped hydrogels and a new layer of Silvadene cream were applied at each dressing change.

On day 10, the same procedure for hydrogel collection, fluid collection, photography, and sensing was followed as on days 3 and 6, then just prior to euthanasia, full thickness skin specimens from the centre of each wound were harvested into buffered formalin for histologic examination of routinely processed hematoxylin and eosin-stained sections. Morphologic parameters for wound healing were scored in a treatment-blinded fashion.

The 0.5% HEMA hydrogels were made by the Gardella group. To load the sterilized hydrogels with keratinocyte growth factor (KGF), lyophilized KGF were reconstituted in sterile PBS. KGF was equilibrated into the gel by diffusion.

Wounds were identified as follows:

A (#8) untreated (lower right)

B (#7) Silvadene cream

C (#6) plain hydrogel

D (#5) KGF-doped hydrogel (upper right)

E (#4) KGF-doped hydrogel (lower left)

F (#3) plain hydrogel

G (#2) Silvadene cream

H (#1) untreated (upper left)

Wounds A – D were created by 20 second exposure; wounds E - H were created by 40 second exposure.

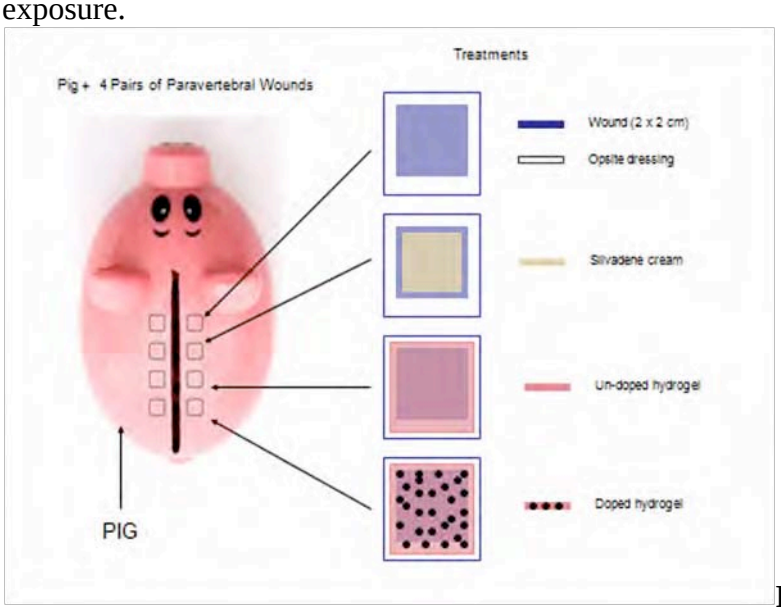


Figure 1 illustrates the template for thermal injury, showing arrangement of treatment groups – untreated, Silvadene cream (standard burn treatment), plain hydrogel, KGF-doped hydrogel. All wounds were then covered with occlusive dressings.

Table 1 shows wound fluid cytokine analysis.

sample	sterile PBS fluid added	O2 Sensor	wound	KGF (pg/mL)	IL-1 (pg/mL)	IL-6 (pg/mL)	IL-12 (pg/mL)	TNF-alpha (pg/mL)
A day 3	20 ul	1	untreated	23 +/- 4	122 +/- 10	< 2	<2	20 +/- 10
B day 3	40 ul	2	silvadene	35 +/- 8	125 +/- 12	<2	<2	25 +/- 2
C day 3	20 ul	3	plain Hydrogel	40 +/- 12	130 +/- 14	<2	<2	30 +/- 6
D day 3	20 ul	4	KGF Hydrogel	390 +/- 10	166 +/- 25	<2	<2	50 +/- 6
E day 3	20 ul	5	KGF Hydrogel	425 +/- 15	175 +/- 13	3 +/- 2	<2	49 +/- 8
F day 3	20 ul	6	plain Hydrogel	29 +/- 6	125 +/- 20	<2	<2	35 +/- 11
G day 3	20 ul	7	silvadene	42 +/- 7	133 +/- 19	<2	<2	33 +/- 7
H day 3	20 ul	8	untreated	28 +/- 5	145 +/- 20	<2	<2	16 +/- 4
A day 6	100 ul	1	untreated	44 +/- 5	56 +/- 9	22 +/- 8	<2	19 +/- 5
B day 6	100 ul	3	silvadene	55 +/- 8	60 +/- 15	11 +/- 7	8 +/-1	44 +/- 12
C day 6	100 ul	4	plain Hydrogel	53 +/- 11	88 +/- 25	35 +/- 18	14 +/- 4	55 +/- 5
D day 6	100 ul	6	KGF Hydrogel	380 +/4	125 +/- 16	49 +/- 12	59 +/- 12	89 +/- 13
E day 6	100 ul	8	KGF Hydrogel	444 +/- 28	144 +/- 29	38 +/- 11	45 +/- 16	81 +/- 5
F day 6	100 ul	9	plain Hydrogel	55 +/- 10	82 +/- 18	22 +/- 6	22 +/- 7	49 +/- 11
G day 6	100 ul	10	silvadene	47 +/- 7	77 +/- 8	28 +/- 12	6 +/2	48 +/- 7
H day 6	100 ul	5	untreated	39 +/- 6	65 +/- 8	28 +/- 11	<2	32 +/- 8
A day 10	20ul	12	untreated	18 +/- 4	28 +/- 18	40 +/- 11	<2	13 +/- 6
B day 10	20ul	13	silvadene	20 +/- 10	30 +/- 7	35 +/- 16	11 +/- 5	12 +/- 8
C day 10	20ul	14	plain Hydrogel	11 +/- 6	44 +/- 18	44 +/- 18	18 +/- 10	24 +/- 5
D day 10	20ul	15	KGF Hydrogel	210 +/- 23	138 +/- 12	59 +/- 6	80 +/- 15	45 +/- 14
E day 10	20ul	16	KGF Hydrogel	189 +/18	116 +/- 23	77 +/- 19	62 +/- 20	39 +/- 8
F day 10	20ul	17	plain Hydrogel	15 +/- 7	56 +/- 12	39 +/- 4	15 +/- 5	18 +/- 6
G day 10	20ul	19	silvadene	18 +/- 8	27 +/- 9	29 +/- 3	26 +/- 9	19 +/- 6
H day 10	20ul	20	untreated	23 +/- 3	35 +/- 7	39 +/- 12	4 +/- 1	11 +/- 3

Uncertainties (+/-) reflect the average of five (5) replicate measurements on each sample by PIXIES and ELISA.

The correlation between ELISA and PIXIES results is 0.952 between 2 (detection limits) and 50 pg/ml.

The data has been corrected for the volume of PBS.

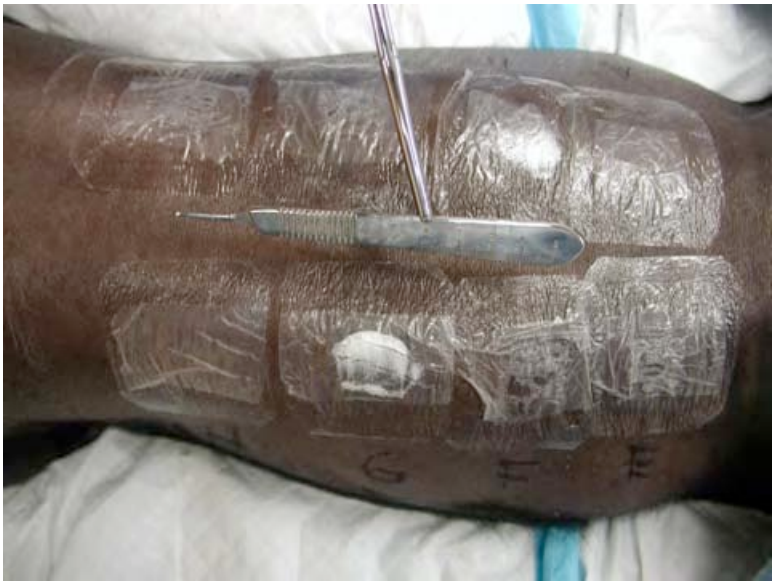


Figure 2 shows arrangement of burns on back of pig and dressings over treatment groups.

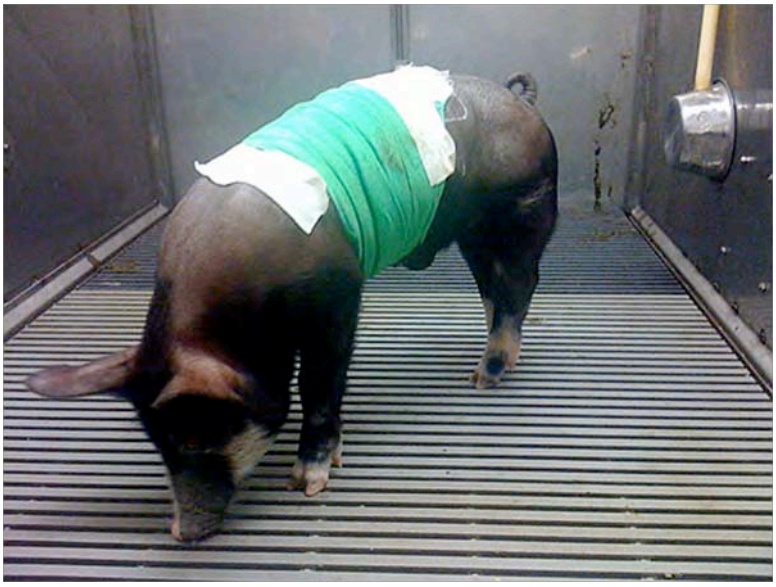


Figure 3 shows the animal post-operatively

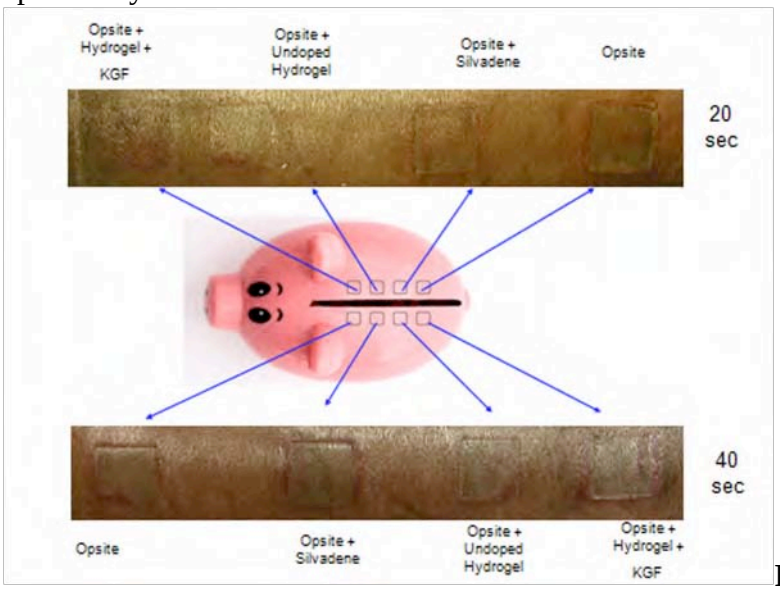


Figure 4 illustrates the effects of the treatment groups on wound healing at day 10 post-injury.

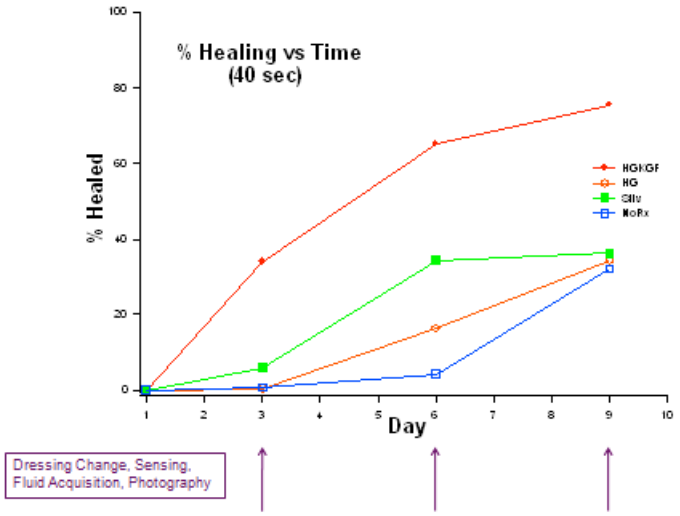


Figure 5 shows the time course of healing expressed as % area healed over time. Areas were determined from analysis of digital photographs.

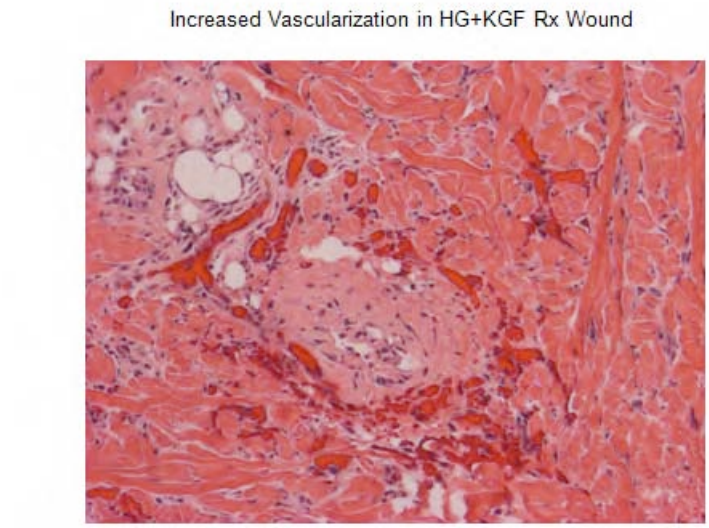


Figure 6 shows cutaneous tissue harvested from KGF-hydrogel-treated wound (day 10). Hematoxylin and eosin stained section revealed increased vascularization of connective tissue beneath the healed wound compared with other treatment groups (not shown).

Phase II

Technical Objective 3:

This objective has been delayed due to the initial difficulties identifying a site for recruitment of subjects with burn wounds.

KEY RESEARCH ACCOMPLISHMENTS

- Reproducible partial-thickness thermal injury could be inflicted in an *in vivo* model system
- Hydrogel dressings could be fabricated and doped with a protein known to be important in wound healing

- KGF could be released from the hydrogel, based on cytokine profile analysis
- KGF released from the hydrogel was active and did enhance healing, based on histological analysis of the tissue and on calculated rate of healing
- Sensing of *in vivo* wounds was possible; in this case oxygen content was determined
- PIXIES and ELISA analysis of wound fluid were highly correlated within the linear range of detection
- Analysis of wound fluid revealed differences in cytokine content and temporal expression between KGF treatment and the other treatment groups

APPENDICES

Appendix A: time tendency in each protein level

1. MMP array

Protein	μ		α_i		β		γ_i	
	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value
MMP-1	6083.81	0.3897	6479.27	.5127	-275.02	.3806	399.49	.3641
MMP-2	2532.01	.3582	-549.12	.8812	20.98	.8191	-105.37	.4025
MMP-3	854.18	.1088	100.96	.8865	-29.97	.2609	43.04	.2489
MMP-8	11464.31	.0731	981.89	.9068	-3.09	.9895	-39.73	.9028
MMP-9	65757.62	.1946	10697.90	.8739	-170.26	.9288	-642.58	.8081
MMP-10	158.76	.0473	-158.39	.1972	1.78	.5292	-1.82	.6417
MMP-13	1495.60	.0956	-1134.77	.3754	-21.20	.6171	32.1763	.5875
TIMP-1	2745.29	.0274	2999.49	.1352	-57.88	.1554	43.43	.4297
TIMP-2	3404.208	.0011	1066.71	.4323	31.17	.3337	4.18	.9238

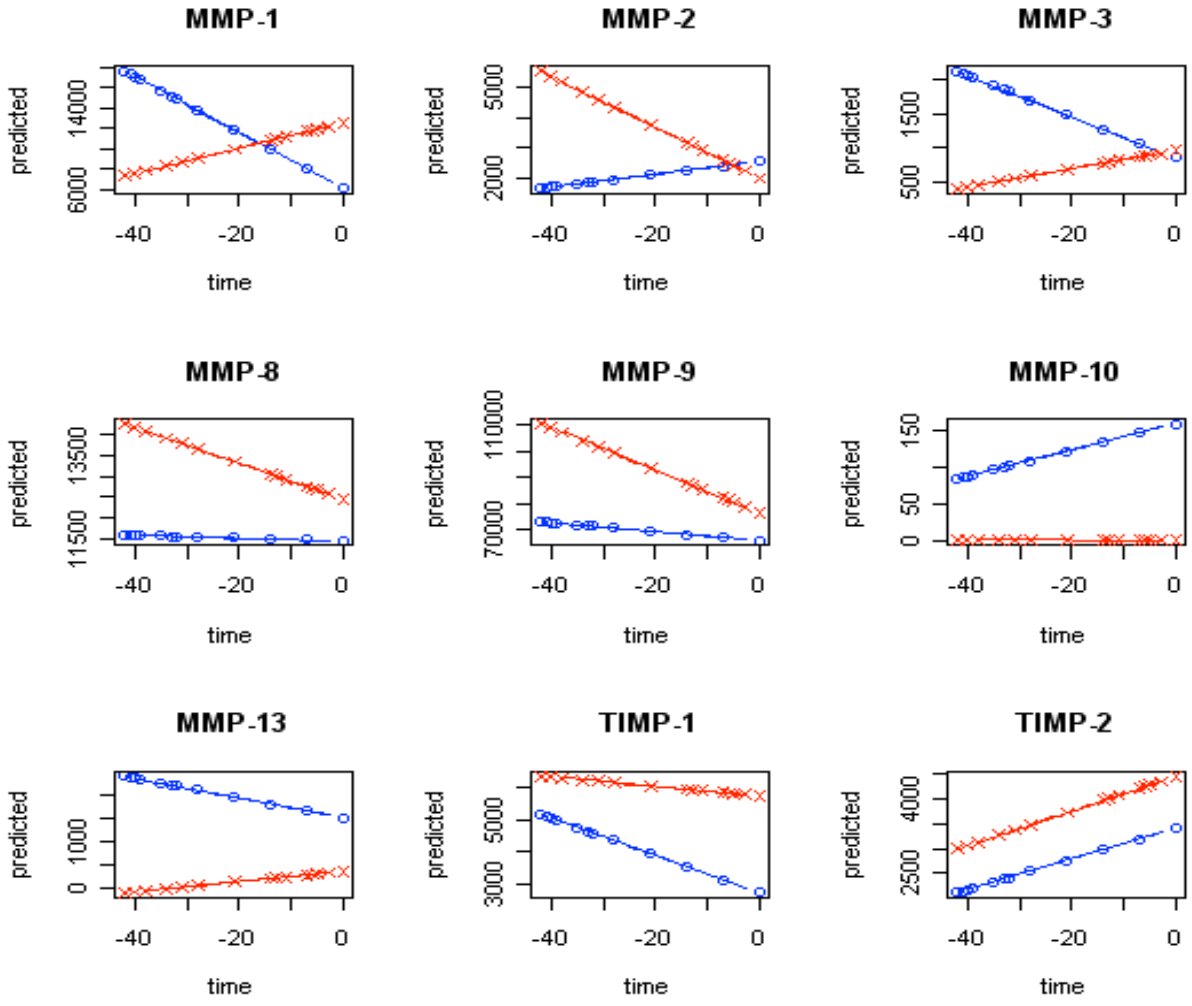
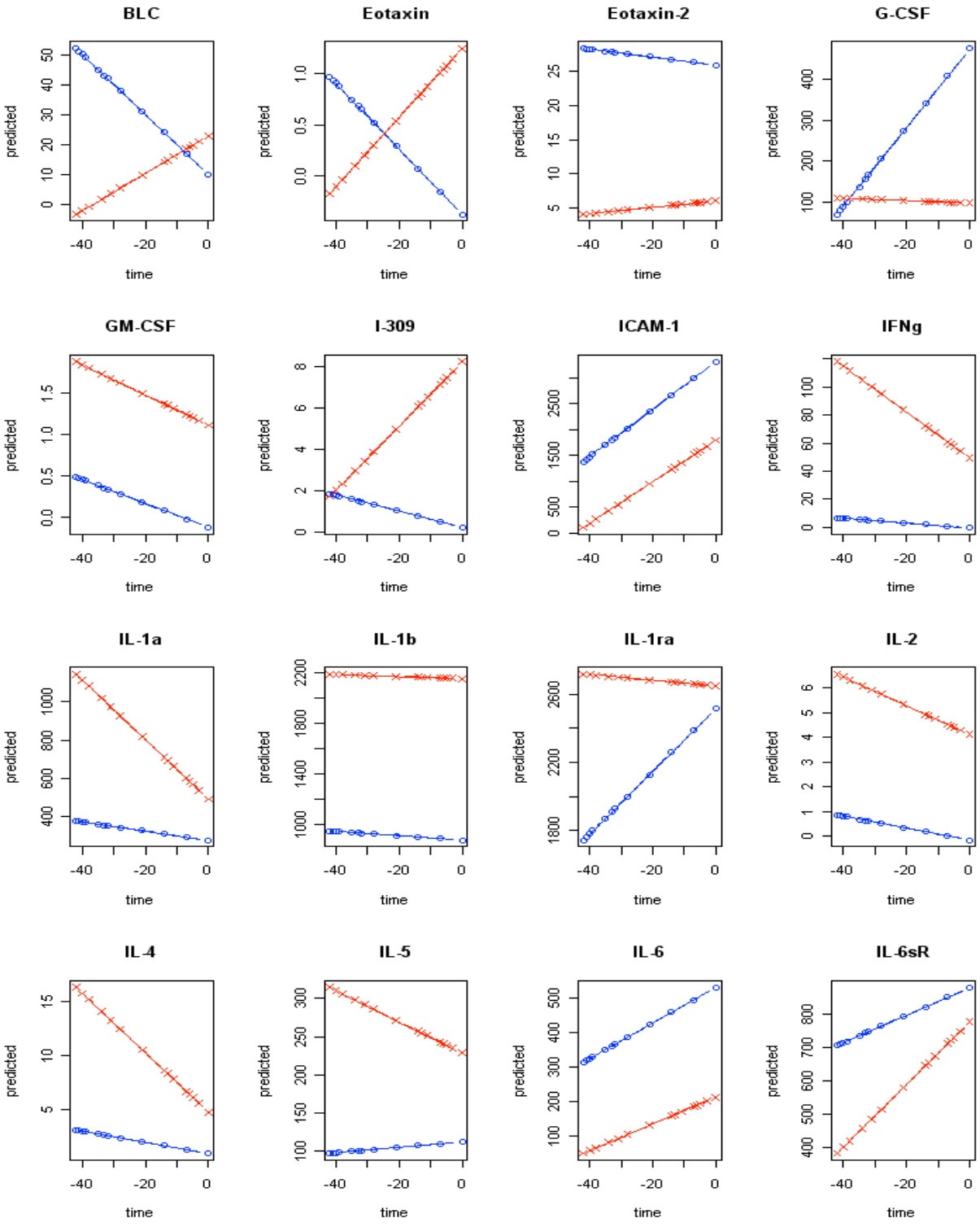


Figure 1 Linear Time Effect (Blue = healed, Red = chronic)

2. HI3 array

Protein	μ		α_i		β		γ_i	
	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value
BLC	9.97	.6766	12.00	.6889	-1.00	.2146	1.63	.1420
Eotaxin	-.37	.7558	1.61	.3531	-.031	.4268	.065	.2349
Eotaxin-2	26.01	.0454	-19.98	.2886	-.057	.8917	.104	.8553
G-CSF	474.30	.0013	-376.48	.1098	9.61	.0405	-9.92	.1164
GM-CSF	-.12	.9090	1.23	.4166	-.014	.7259	-.0036	.9491
I-309	.24	.9280	8.00	.0921	-.039	.6608	.19	.1169
ICAM-1	3306.79	.0000	-1514.83	.1671	46.00	.0758	-5.716	.8697
IFNg	-.38	.9956	49.65	.6064	-.16	.9441	-1.47	.6464
IL-1a	273.17	.2838	215.45	.5410	-2.38	.8132	-13.28	.3481
IL-1b	873.41	.1456	1278.81	.1787	-1.70	.9441	.8492	.9798
IL-1ra	2515.16	.0333	135.4608	.9293	18.38	.6308	-20.02	.7012
IL-2	-.15	.9789	4.27	.5904	-.024	.9093	-.034	.9070
IL-4	.95	.9228	3.81	.7739	-.051	.8762	-.22	.6195
IL-5	111.77	.6093	116.72	.6943	.36	.9621	-2.31	.8170
IL-6	529.7629	.0261	-316.32	.3453	5.15	.5405	-1.29	.9111
IL-6sR	878.97	.0003	-102.93	.7281	4.12	.5742	5.2125	.6026
IL-7	5.64	.8788	21.46	.6708	-.096	.9442	-.54	.7759
IL-8	821.5348	.0677	1411.30	.0795	-2.46	.8668	16.61	.4091
IL-10	.07	.9289	2.02	.8152	-.074	.7497	-.11	.7191
IL-11	86.43	.6395	320.01	.2439	.7824	.8991	4.12	.6257
IL-12p40	9.15	.8116	5.21	.9200	.22	.9031	-2.37	.3416
IL-12p70	-.82	.5853	1.04	.6103	-.078	.1266	.083	.2264
IL-13	7.07	.5743	8.51	.6217	-.073	.8723	-.018	.9769
IL-15	.00	1.0000	11.60	.2467	.00	1.0000	.040	.8943
IL-16	1227.88	.0008	-795.94	.1499	21.32	.0646	-11.32	.4619
IL-17	24.42	.4516	44.52	.3483	.0034	.9980	.21	.9143
MCP-1	308.00	.0065	-75.32	.6069	6.99	.0570	-2.42	.6197
MCSF	23.31	.3973	-2.76	.9409	-.98	.5080	-.19	.9255
MIG	3540.28	.0001	-3321.23	.0457	97.49	.0011	-101.94	.0096
MIP-1a	38.92	.7410	109.08	.5070	.55	.8883	3.32	.5379
MIP-1b	52.49	.5880	85.05	.5273	1.03	.7507	2.36	.5947
MIP-1d	4.29	.6864	1.42	.9215	-.78	.2523	.71	.4493
PDGF-BB	.058	.9680	.86	.6726	-.018	.9136	-.14	.5316
TIMP-1	1760.77	.1807	4152.23	.0816	-86.58	.1063	91.52	.2158
TIMP-2	1812.56	.0437	2719.53	.0874	-20.64	.6262	16.54	.7811
TNFa	10.52	.4558	10.25	.5957	.18	.7033	-.14	.8225
TNFb	-19.60	.9700	193.09	.7835	-8.73	.6259	-2.13	.9308
TNF RI	3387.43	.0157	2449.27	.2347	54.90	.2394	-62.44	.3267
TNF RII	1618.52	.0069	26.87	.9717	27.67	.1644	-53.02	.0554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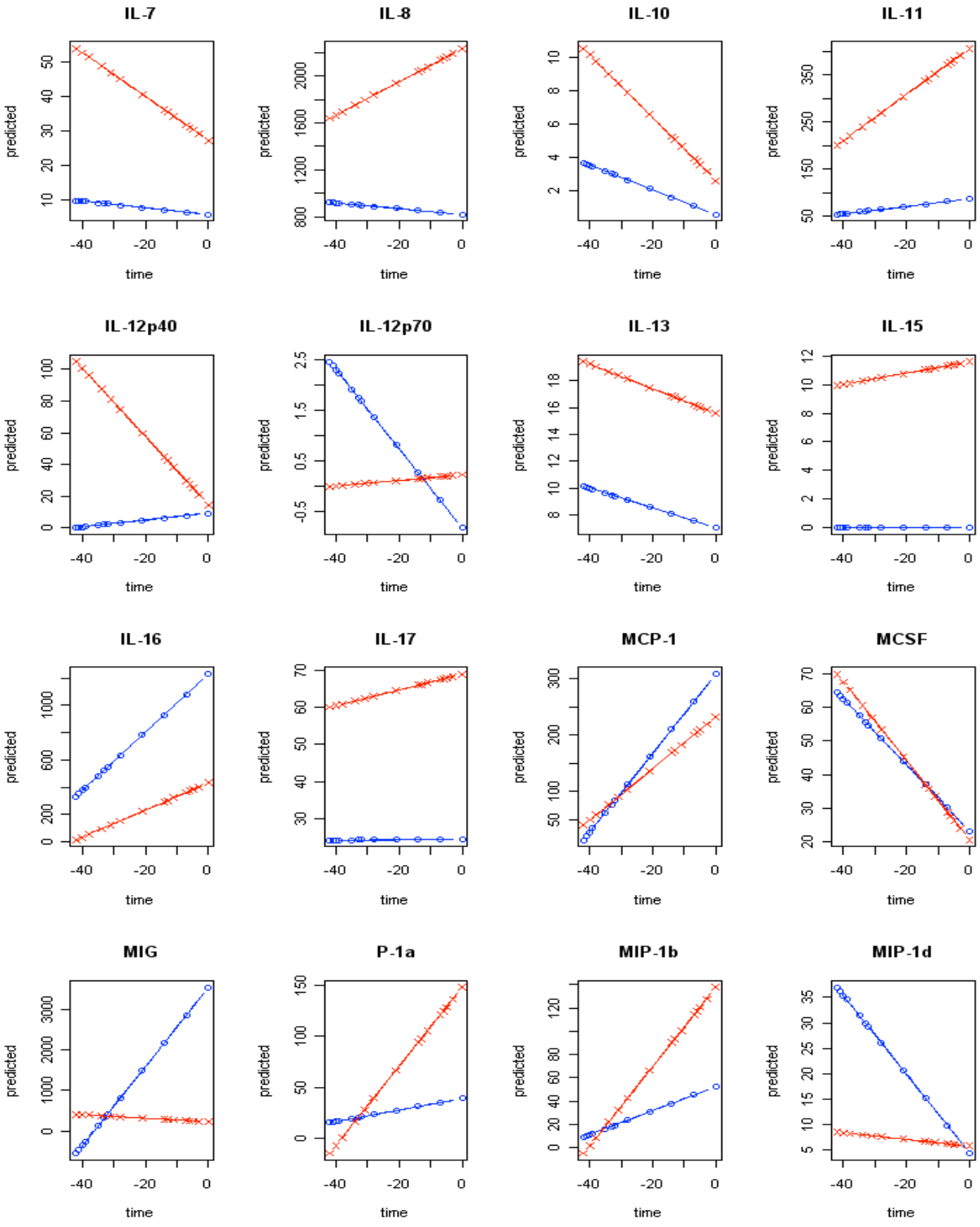
IL-5
IL-6
IL-6sR
predicted
time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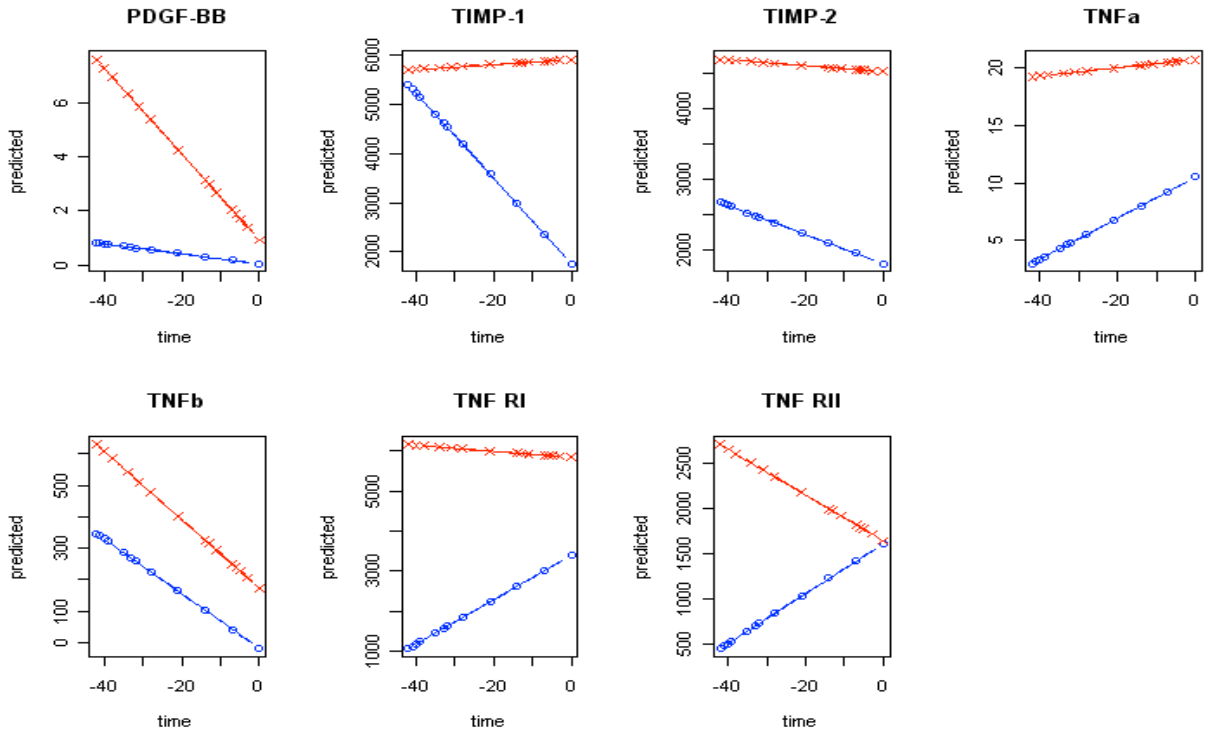
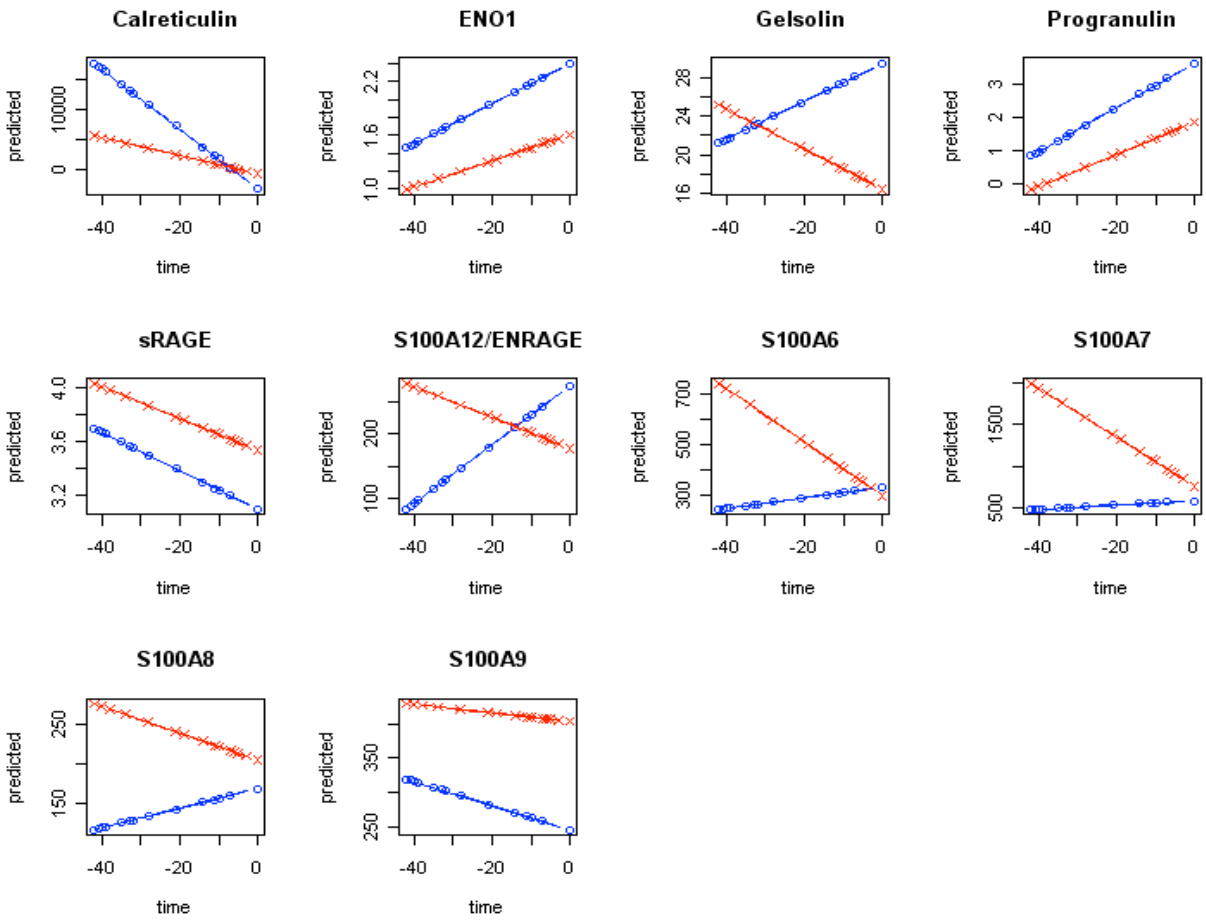
time



3. Custom array

Protein	μ		α_i		β		γ_i	
	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value
Calreticulin	-3320.52	.5804	2550.17	.7507	-504.76	.1515	352.65	.4678
ENO1	2.41	.0772	-.80	.6518	.023	.6303	-.0081	.9028
Gelsolin	29.47	.0872	-13.04	.5660	.20	.7444	-.40	.6368
Progranulin	3.67	.0282	-1.80	.4151	.068	.2398	-.019	.8153
sRAGE	3.10	.2720	.44	.9052	-.01	.8842	.0025	.9857
S100A12/ENRAGE	274.05	.0990	-96.36	.6579	4.54	.4310	-6.92	.3966
S100A6	333.72	.2014	-33.31	.9223	2.11	.8170	-12.61	.3312
S100A7	582.97	.4269	178.19	.8540	2.45	.9241	-31.84	.3851
S100A8	168.94	.1959	36.01	.8333	1.22	.7892	-2.88	.6547
S100A9	245.63	.3408	158.09	.6447	1.76	.8455	1.15	.9281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0
time
ENO1
predicted
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1.6
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-20
0
time
Gelsolin
predicted
16
20
24
28
-40
-20
0
time
Progranulin
predicted
0
1
2
3
-40
-20
0
time
sRAGE
predicted
3.2
3.6
4.0
-40
-20
0
time
S100A12/ENRAGE
predicted
100
200
-40
-20
0
time
S100A6
predicted
300
500
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time